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Differences between Brahman and Holstein cows in response to estrus synchronization, superovulation and resistance of embryos to heat shock☆

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Abstract

Embryos from *Bos indicus* are more resistant to elevated culture temperature (i.e. heat shock) than embryos from some *Bos taurus* breeds. The present experiment was designed to determine if Brahman embryos have greater resistance to heat shock than Holstein embryos at a stage in development before the embryonic genome was fully activated. A second objective was to test breed effects on estrus synchronization and superovulation responses. A total of 29 Brahman and 24 Holstein cows were subjected to estrus synchronization using gonadotropin releasing hormone (GnRH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) superovulation. Embryos were collected at 48 h and day 5 after insemination. There was a tendency for a lower proportion of Brahmans to be detected in standing estrus than Holsteins. There were no differences between breeds in the proportion of cows detected in estrus using both tailpaint and standing estrus as criteria or in interval from $PGF_{2\alpha}$ to estrus. The degree of synchrony in estrus was greater for Brahmans. Superovulation response was generally similar between breeds. At 48 h after insemination, there was a tendency for a greater proportion of Brahman oocytes to have undergone cleavage. Uncleaved oocytes were cultured for an additional 24 h—at this time, cleavage rate was similar between breeds. When embryos reached the 2–4-cell stage, they were heat-shocked for 4.5 h at 41 °C. This heat shock reduced the proportion of embryos that developed to the blastocyst stage but there was no breed × treatment interaction. At day 5 after insemination, the number of embryos recovered was too low to allow comparison of breed effects. In conclusion, genetic effects on cellular thermotolerance that make Brahman embryos more resistant to heat shock are not expressed at the 2–4-cell stage. There were few differences

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between Brahman and Holstein in response to estrus synchronization and superovulation. The fact that cleavage tended to occur earlier in Brahman than Holstein embryos suggests breed differences in timing of ovulation, fertilization or events leading to cleavage.

Keywords: Brahman; Holstein; Superovulation; Estrus synchronization; Heat shock; Embryo

1. Introduction

The superior thermoregulatory ability of *Bos indicus* cattle makes these animals better adapted for hot climates than many *Bos taurus* breeds. Thus, *B. indicus* cattle are less likely to undergo hyperthermia in response to heat stress than *B. taurus* (Finch, 1986; Hammond et al., 1998). At the cellular level, there is evidence that cells from Brahman cattle have superior resistance to exposure to elevated temperature. Such genetic differences have been reported for endometrium (Malayer and Hansen, 1990) and lymphocytes (Kamwanja et al., 1994; Paula-Lopes et al., 2003). Recently, it was observed that exposure of 9–16-cell in vitro-produced embryos to 41 °C for 9 h inhibited development of Brahman embryos less than Angus or Holstein embryos (Paula-Lopes et al., 2003). This result implies that the deleterious effect of heat stress on fertility, which in part depends upon inhibition of embryonic development (Putney et al., 1988; Ealy et al., 1993), can be affected by embryonic genotype. Nonetheless, the embryonic genome in cattle does not become fully activated until the 8–16-cell stage (Memili and First, 2000) and it is possible that genetic effects on embryonic resistance to elevated temperature are not seen at stages of development before the 8–16-cell stage.

There are other differences in reproductive physiology between *B. indicus* and *B. taurus*, with the former having reduced duration of estrus (Plasse et al., 1970; Rea et al., 1999), a shorter period from onset of estrus to the LH surge as well as from the LH surge to ovulation (Randel, 1984), increased number of follicles (Alvarez et al., 2000), and higher plasma concentrations of insulin-like growth factor I (Simpson et al., 1994; Alvarez et al., 2000). Reports are inconsistent regarding breed differences in superovulation responses (Cordova-Santamaria and Fraga-Escamilla, 1991; Massey and Oden, 1984; Munro, 1986; Simpson et al., 1994).

The present experiment was designed to determine if Brahman embryos have greater resistance to heat shock than Holstein embryos at a stage in development before the embryonic genome was fully activated. A second objective was to test breed effects on estrus synchronization and superovulation responses.

2. Materials and methods

2.1. Cows

The study was conducted in autumn 2000 with a total of 53 non-lactating cows (24 Holstein and 29 Brahman). Cows were palpated per rectum before the study to determine

reproductive soundness and status and were moved to a common facility 14 days prior to the beginning of the study. Cows were maintained on fall and winter-grass pasture and received supplemental hay, molasses, and minerals for the duration of the study. Body weight and body condition score (Long et al., 1979) were measured at the time of first injection of gonadotropin releasing hormone (GnRH).

2.2. Estrus synchronization

The experiment was conducted in five replicates. For each replicate, a group of four to six cows in each breed were subjected to estrus synchronization; four of these cows from each group were selected for superovulation based on estrus detection or rectal palpation of a corpus luteum. Two Brahman cows that where not used for superovulation in early replicates were subjected to estrus synchronization again in replicate 5 so that the total number of observations for estrus synchronization are 24 for Holstein and 31 for Brahman.

Estrus synchronization was accomplished by i.m. injections of 100 μg GnRH (Cystorelin, Rhone Merieux, Athens, GA) on day 0, and 12.5 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, Pharmacia and Upjohn, Kalamazoo, MI) on days 7 and 8. Estrus was detected each morning and afternoon from days 9 to 12. A tailpaint system modified from that described by Macmillan et al. (1988) was employed to aid in estrus detection. Livestock marking chalk was applied to the tail head of each animal. The tailpaint was examined twice daily and scored from 0 to 3, where 3 represents undisturbed paint and 0 represents the situation when little or no tailpaint remained. A cow was considered in estrus when tailpaint score was first below 0.5.

2.3. Superovulation

Superovulation was initiated on day 19 (day 0 = day of GnRH injection for estrus synchronization) through multiple injections, i.m., of follicle stimulating hormone (FSH; Folltropin-V, Vetrepharm, London, Ont., Canada). Injections were given at 12 h intervals in gradually decreasing doses (two injections of 40 mg, four of 30 mg, and two of 20 mg). To induce luteal regression, 25 mg $PGF_{2\alpha}$ was injected i.m., coincident with the sixth injection of FSH and an additional 15 mg was injected at the time of the seventh FSH injection. Cows were artificially inseminated using semen from bulls of the same breed at 24 and 36 h after administration of the final injection of FSH. For each replicate, all cows within a breed were bred to the same sire; a different sire was used for each replicate. At the time of first insemination, 100 μg GnRH was injected i.m. to facilitate ovulation.

2.4. Embryo collection

Cows were randomly assigned within breed and replicate to be flushed at either 48 h or day 5 after first insemination. To collect embryos at 48 h after insemination, cows were sacrificed and reproductive tracts removed. The oviducts were flushed using 10 ml of a flushing medium composed of 10 mM sodium phosphate, pH 7.4, containing 0.9% (w/v)

NaCl, 2% (v/v) bovine steer serum (Pel-Freez Biologicals, Rogers, AK), 100 U/ml penicillin and 100 μg/ml streptomycin. At the time of embryo collection, ovulation fossa on the ovaries were counted. Embryos were collected at day 5 (∼120 h after first insemination) by standard transcervical, non-surgical embryo recovery using a Foley catheter. The uterus of each donor was flushed using 1 l of pre-warmed flushing medium. The flushings were passed though an EM-Con filter (75 μm pore size, Agtech, Manhattan, KS) and the concentrated flushing searched under the microscope to identify embryos.

2.5. Embryo culture and heat shock

For each replicate, embryos collected at 48 h after first insemination were pooled by breed and cultured in 25 μl microdrops of modified KSOM (Paula-Lopes et al., 2003) for 24 h at 38.5 °C in 5% (v/v) CO_2 in humidified air. Embryos that were between the 2- and 4-cell stages after culture were then separated, divided into two groups of equal number, placed in fresh 25 μl microdrops and subjected to one of two treatments. Control embryos were cultured at 38.5 °C continuously while heat-shocked embryos were placed in a 41 °C incubator containing 7% (v/v) CO_2 in humidified air for 4.5 h and then were returned to the 38.5 °C incubator. The higher CO_2 content at 41 °C in humidified air was used to ensure that pH was similar at 38.5 and 41 °C (Rivera and Hansen, 2001). Stage of development was measured at days 7 and 8 relative to first insemination.

For each replicate, embryos collected at day 5 were pooled by breed and cultured in modified KSOM for 2 h at 38.5 °C to adjust embryos to the culture environment. Then, embryos >16 cells in number were separated, divided into two groups of equal number, placed in fresh 25 μl microdrops and subjected to control or heat shock treatments as described above.

2.6. Statistical analysis

Data were analyzed by using least-squares analysis of variance using the GLM procedure of SAS (1989). For data characterized by one observation per cow, the mathematical model included breed, replicate, and breed × replicate. In some analyses, body condition score and body weight were used as a covariate. Other analyses were performed using body condition score class (body condition score ≤5.5 versus >5.5) as a discrete variable in the analysis. For embryo culture data, the mathematical model included effects of breed, replicate, cow pair, treatment, and all interactions. The number of embryos cultured per drop was used as a covariate. Cleavage data was evaluated at the time of embryo collection and after 24 h of culture. Accordingly, the effect of time and interactions with time were also included in the model. Percentage data (percent cleaved, percent developed) were calculated for each cow pair (the pair of cows within a breed at each replicate used for embryo collection). Data were analyzed with and without arcsine transformation. Reported probability values are from the analysis of the transformed data while least-squares means ± S.E.M. are from the analysis of untransformed data. Cow pair was considered a random effect and other main effects were considered fixed. Tests of significance were made using the appropriate error term calculated from expected mean squares.

3. Results

3.1. Response to synchronization

Brahman cows weighed less ($P < 0.001$) at the initiation of the estrus synchronization protocol than Holstein cows but both breeds had similar body condition scores (Table 1). There was a tendency ($P = 0.08$) for a lower proportion of Brahman cows to be detected in estrus using visual detection than Holsteins (Table 1). When tailpaint was included as an additional criterion for estrus detection, there was no difference between breeds in the proportion of cows detected in estrus. There was also no difference between breeds in the interval between the first $PGF_{2\alpha}$ injection and when cows were detected in estrus (Table 1).

The distribution of intervals from first injection of $PGF_{2\alpha}$ to detection of estrus is shown in Fig. 1. There were two lines of evidence that estrus was more synchronous in Brahman cows than in Holstein cows. The first is based on calculation of the degree of synchrony of estrus, defined as the proportion of cows detected in estrus between 2.5 and 3.5 days after first $PGF_{2\alpha}$ injection. Using both behavioral estrus and tailpaint as criteria to detect estrus, there was a higher ($P < 0.05$) proportion of Brahman cows successfully synchronized as compared to Holstein cows (Table 1). Though non-significant, the same tendency was apparent for estrus based on visual observation only. The second line of evidence was that

Table 1
Differences between Holstein and Brahman cows in estrous response to synchronization with GnRH and $PGF_{2\alpha}$

	Brahman	Holstein
Body weight at initiation of synchrony (kg)[a]	528.0 ± 17.0	677.0 ± 20.1***
Body condition score at initiation of synchrony[a,b]	5.5 ± 0.18	5.5 ± 0.20
Percent and proportion of cows observed in estrus		
Standing estrus	29 (9/31)	50 (12/24)†
Standing and tailpaint	58 (18/31)	71 (17/24)
Interval from first $PGF_{2\alpha}$ injection (days) to estrus		
Standing estrus[a]	2.9 ± 0.29	3.4 ± 0.25
Standing and tailpaint[a]	3.2 ± 0.15	3.5 ± 0.15
Degree of estrus synchrony[c]		
Standing estrus	100 (9/9)	75 (8/12)
Standing and tailpaint	83 (15/18)	53 (9/17)*
Standard deviation (days), interval from first $PGF_{2\alpha}$ injection to estrus		
Standing estrus	0.43	1.08**
Standing estrus and tailpaint	0.77	0.78

[a] Data are least-squares means ± S.E.M.
[b] 1–10 scale.
[c] Data represent the percent and proportion of estrous cows that were observed in estrus between 2.5 and 3.5 days after first injection of $PGF_{2\alpha}$.
† $P = 0.08$.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

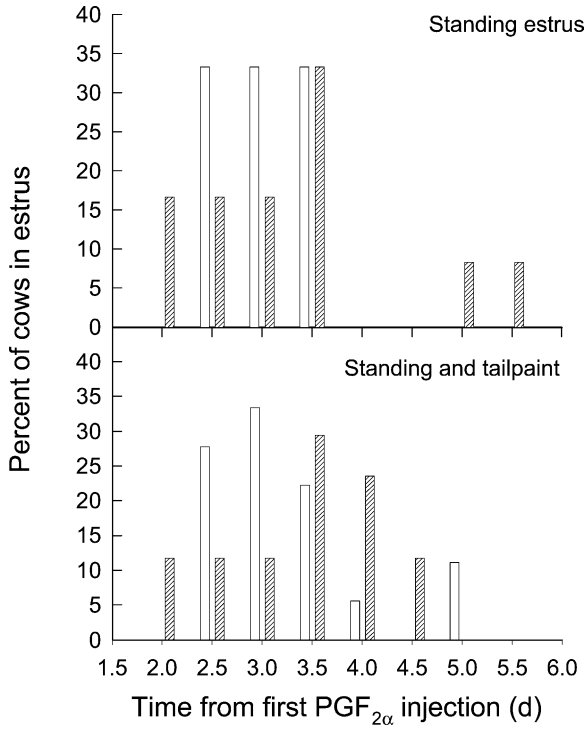

Fig. 1. Distribution of interval from first injection of $PGF_{2\alpha}$ to estrus for Brahman (open bars) and Holstein (hatched bars) cows. Estrus was based on visual observation of standing estrus only (top panel; $n = 9$ Brahman and 12 Holstein) or by a combination of standing estrus and tailpaint scores (bottom panel; $n = 18$ Brahman and 17 Holstein).

the variation in interval from first injection of $PGF_{2\alpha}$ to estrus based on visual observation only was lower ($P < 0.01$) for Brahman cows (Table 1).

There was no relationship between either body weight or body condition score and estrus responses after synchronization. This was true when body condition score and weight were used as covariates and when cows were divided into those with body condition score $\leq$5.5 versus those with body condition score >5.5 (results not shown).

3.2. Superovulation response

Results on superovulation response for cows slaughtered at 48 h after first insemination are shown in Table 2. There were no breed differences for any trait related to superovulation including cell number at time of recovery, ovulation rate, percent ovulations on right ovary, number of embryos and oocytes recovered, and percent recovery (number of embryos and oocytes recovered/total ovulations).

At the time of oocyte recovery (48 h after insemination), a majority of oocytes had not cleaved. All oocytes and embryos were cultured for 24 h before selecting 2–4-cell embryos

Table 2
Comparison between Holstein and Brahman cows in superovulation response following FSH (collection at 48 h after first insemination)[a]

	Brahman	Holstein
Ovulation rate	15.2 ± 4.14	16.6 ± 4.14
Percent ovulations on right ovary	47.0 ± 6.9	38.0 ± 6.9
Embryos recovered (number)	9.3 ± 2.93	10.9 ± 2.93
Embryos recovered (percent of ovulations)	66.0 ± 9.6	69.0 ± 9.6
Embryo cell number at recovery	1.6 ± 0.44	1.2 ± 0.53

[a] Data represent least-squares means ± S.E.M. for 10 cows per group.

for heat shock. During this time, many oocytes that had not cleaved earlier at the time of collection had undergone cell division. There was a tendency for a breed effect on the timing of the first cleavage division (Fig. 2). In particular, the probability value for the breed × time interaction was $P = 0.09$. This interaction represented the fact that at the time of collection (i.e. 48 h after insemination), a greater proportion of oocytes had cleaved for Brahman cows than for Holstein cows. At 24 h after collection (72 h after insemination and after 24 h of culture), cleavage rate was similar between breeds.

For cows flushed at day 5 after insemination, embryos and oocytes were recovered from only 4 of 10 Brahman cows and 3 of 10 Holstein cows. A total of 17 embryos and oocytes were recovered (10 Brahman and 7 Holstein), with 16 embryos classified as being >16 cells in number and 1 (Holstein) being classified as either an unfertilized oocyte or degenerate embryo.

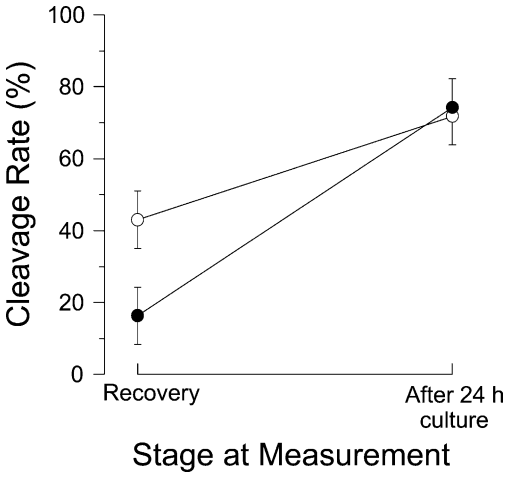

Fig. 2. Timing of cleavage in Brahman (open circle) and Holstein embryos (closed circle) recovered from the reproductive tract at 48 h after insemination. Cleavage rate was examined at the time of collection (48 h after insemination) and after oocytes and embryos had been cultured for 24 h. Data represent least-squares means ± S.E.M. for five pairs of cows for each breed.

Table 3
Development of embryos from Holstein and Brahman cows at day 8 after insemination following heat shock (41 °C for 4.5 h) administered at the 2–4-cell stage

Breed	Treatment	Percent and proportion of embryos developing to[a]		
		>8-Cell stage	Morula	Blastocyst
Holstein	Control	26 ± 13.2 (12/35, 34)	22 ± 5.7 (11/35, 31)	16 ± 5.0 (9/35, 26)
Holstein	Heat shock	0 ± 10.8 (0/38, 0)	0 ± 4.7 (0/38, 0)	0 ± 5.3 (0/38, 0)
Brahman	Control	32 ± 13.7 (10/29, 35)	32 ± 6.0 (10/29, 35)	13 ± 5.2 (4/29, 14)
Brahman	Heat shock	27 ± 14.1 (3/26, 12)	7 ± 6.1 (2/26, 8)	0 ± 5.3 (0/26, 0)
Probability				
Breed		0.09	0.09	>0.10
Treatment		<0.01	<0.01	<0.05
Breed × treatment		>0.10	>0.10	>0.10

[a] Data outside parentheses represent the least-squares means ± S.E.M. (in percentage). Data inside parentheses represents the proportion and percent of embryos that developed.

3.3. *Responses of embryos to heat shock*

Exposure of 2–4-cell embryos to 41 °C for 4.5 h reduced the proportion of embryos that developed to >8-cell ($P < 0.01$), morula ($P < 0.01$), and blastocyst stages ($P < 0.05$) at day 8 after insemination (Table 3). Heat shock reduced development in both breeds and there was no breed × temperature interaction affecting development to the blastocyst stage. There was a tendency ($P = 0.09$) for breed effects on the proportion of embryos reaching the >8-cell and morula stages of development, with more Brahman embryos reaching these developmental endpoints than Holstein embryos.

A total of 10 embryos classified as being >16 cells (6 Brahman and 4 Holstein) that were collected at day 5 were used for the heat shock experiment. Development to blastocyst was 100% for embryos at 38.5 °C (2/2 Brahman and 2/2 Holstein) and 83.3% for embryos at 41 °C (3/4 Brahman and 2/2 Holstein).

4. Discussion

While several studies indicate that cells and embryos from Brahman cattle are more resistant to heat shock than materials from Angus and Holstein cattle (Malayer and Hansen, 1990; Kamwanja et al., 1994; Paula-Lopes et al., 2003), such a breed difference was not seen for 2–4-cell embryos in this experiment. In contrast, Brahman embryos ≥9 cells in development were more resistant to heat shock than Holstein or Angus embryos at a similar stage of development (Paula-Lopes et al., 2003). Perhaps, the most likely explanation for the discrepancy in results is the difference in stage in development when heat shock was applied. Since the embryonic genome is not fully activated until the 8–16-cell stage (Memili and First, 2000), it is possible that some of the genes conferring cellular resistance to heat shock in Brahmans are not expressed at the 2–4-cell stage. While the 2-cell embryo can undergo some gene activation in response to heat shock, for example, increased transcription

of the *hsp70* gene (Chandolia et al., 1999), it is unlikely that the complete array of thermoprotective responses occur at the 2-cell stage. For example, the induced thermotolerance response, which occurs in embryos >8-cell stage (Ealy and Hansen, 1994; Paula-Lopes and Hansen, 2002a), does not occur in 2-cell embryos (Al-Katanani and Hansen, 2002). Similarly, apoptosis responses, which confer some resistance to heat shock (Paula-Lopes and Hansen, 2002a), do not occur until after the 8-cell stage (Paula-Lopes and Hansen, 2002b).

There are two other possible reasons why there was a failure to note breed effects on embryonic resistance to heat shock at the 2–4-cell stage. The heat shock used, which nearly completely blocked development, may have been too severe to allow breed differences to be expressed. In addition, 2–4-cell embryos in the present study underwent oocyte maturation, fertilization and very early development in vivo whereas embryos used in the study of Paula-Lopes et al. (2003) were produced entirely in vitro. A number of biochemical and morphological differences are known to exist between bovine embryos produced in vivo and in vitro (Thompson, 1997; Boni et al., 1999) and one or more of these may have affected breed differences in cellular resistance to heat shock. At least in the mouse, culture itself can modify embryonic responses to heat shock (Ealy and Hansen, 1994).

Originally, it was planned to determine differences between breeds in embryonic sensitivity to heat shock at two stages of development: the 2–4-cell stage (collected 48 h after insemination) and the morula stage (collected at day 5 after insemination). However, the poor recovery of embryos at day 5 precluded a test of whether stage of development determined the magnitude of breed differences in resistance to heat shock. Embryos are believed to enter the uterus by day 5 after estrus (Betteridge and Fléchon, 1988). Given that most ova had not cleaved at 48 h after first insemination, it is likely that many of the embryos at day 5 after insemination were still in the oviduct and unrecoverable via the non-surgical procedure for embryo collection that was used. While the low numbers of embryos at day 5 make any conclusions prohibitive, the effect of heat shock on development of embryos at day 5 was much less than for the 2–4-cell embryos. Such a result, using in vivo-produced embryos, is similar to what has been observed for embryos produced in vitro (Edwards and Hansen, 1997; Paula-Lopes and Hansen, 2002b) and is consistent with the idea that embryos become more resistant to heat shock as they proceed through development. The small numbers of embryos recovered at day 5 means that these observations should be repeated before conclusions be reached about developmental changes in resistance to heat shock in embryos developing in vivo.

Estrus is shorter in duration in Brahman cattle than in several *B. taurus* breeds (Plasse et al., 1970; Rea et al., 1999) and, as expected, there was a tendency for a smaller proportion of Brahman cows to be detected in standing estrus than for Holstein cows. This effect of breed represents differences in estrous behavior and not in cyclicity because breed differences in the proportion of cows detected in estrus was reduced when tailpaint scores were also considered.

The synchronization protocol used in the present experiment has been termed Select Synch (Geary et al., 1999). The response of Brahman cows to this protocol was not inferior to that of Holstein cows and, in at least one criterion, degree of synchrony, was superior to Holsteins. One modification to the synchronization protocol as compared to other studies

(Geary et al., 1999; Stevenson et al., 2000) was the manner in which prostaglandin was administered. Previous work indicated that administration of the luteolytic dose of $PGF_{2\alpha}$ as two injections of 12.5 mg at 24 h intervals rather than as a single injection of 25 mg provides more effective luteolysis in Brahman cattle than a single injection (Santos et al., 1988). Accordingly, a split-dose of $PGF_{2\alpha}$ was given in the current experiment.

The literature is inconsistent regarding differences between *B. indicus* and *B. taurus* in superovulation response (Cordova-Santamaria and Fraga-Escamilla, 1991; Massey and Oden, 1984; Munro, 1986; Simpson et al., 1994). Some of the variation between studies could reflect differences in superovulation protocols, breeds used for the *B. indicus* versus *B. taurus* comparison, as well as non-genetic differences between groups of cows. In the present study, the superovulation response was generally similar for Brahman and Holstein cows. Such a finding is a further indication of the applicability of at least some reproductive protocols designed in *B. taurus* for *B. indicus*.

One difference observed between Brahman and Holstein cows was that there was a tendency for Brahman embryos to cleave sooner than Holstein embryos. Such a finding is based on the observation that at the time of collection (48 h after insemination), when cleavage had not yet been finalized, the proportion of ova that cleaved was greater for Brahmans. After culture for 24 h to allow other fertilized ova to cleave, cleavage rate was similar between Brahman and Holstein. Thus, the difference in cleavage rate at 48 h after insemination represents a breed difference in the timing of cleavage rather than in the ability of oocytes to become fertilized. One possibility is that ovulation occurred sooner relative to insemination for Brahmans. As compared to Herefords and Brahman × Herefords, Brahman females have been reported to have a LH surge that occurs earlier in relation to the beginning of estrus and to ovulate sooner after the LH surge (Randel, 1984). Results from in vitro experiments have made it clear that there is considerable variation between embryos in the timing of cleavage and that early cleaving embryos are more likely to develop to the blastocyst stage and survive freezing (Dinnyes et al., 1999; Lonergan et al., 1999). However, there was no difference in development rate to the blastocyst stage between Brahman and Holstein embryos and, therefore, the early cleaving in vivo embryos may not have been superior in developmental potential.

In conclusion, genetic effects on cellular thermotolerance that make Brahman embryos more resistant to heat shock are not expressed at the 2–4-cell stage. There were few differences between Brahman and Holstein in response to estrus synchronization and superovulation, indicating that the protocols used can be effective in both breeds. The fact that cleavage tended to occur earlier in Brahman than Holstein embryos suggests breed differences in timing of ovulation, fertilization or events leading to cleavage.

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